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Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the Coastal and Estuarine Waters of Louisiana, Maryland, Mississippi, and Washington (United States)

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***Vibrio parahaemolyticus* and *Vibrio vulnificus*, which are native to estuaries globally, are agents of seafood-borne or wound infections, both potentially fatal. Like all vibrios autochthonous to coastal regions, their abundance varies with changes in environmental parameters. Sea surface temperature (SST), sea surface height (SSH), and chlorophyll have been shown to be predictors of zooplankton and thus factors linked to vibrio populations. The contribution of salinity, conductivity, turbidity, and dissolved organic carbon to the incidence and distribution of *Vibrio* spp. has also been reported. Here, a multicoastal, 21-month study was conducted to determine relationships between environmental parameters and *V. parahaemolyticus* and *V. vulnificus* populations in water, oysters, and sediment in three coastal areas of the United States. Because ecologically unique sites were included in the study, it was possible to analyze individual parameters over wide ranges. Molecular methods were used to detect genes for thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), and *tdh*-related hemolysin (*trh*) as indicators of *V. parahaemolyticus* and the hemolysin gene *vvhA* for *V. vulnificus*. SST and suspended particulate matter were found to be strong predictors of total and potentially pathogenic *V. parahaemolyticus* and *V. vulnificus*. Other predictors included chlorophyll *a*, salinity, and dissolved organic carbon. For the ecologically unique sites included in the study, SST was confirmed as an effective predictor of annual variation in vibrio abundance, with other parameters explaining a portion of the variation not attributable to SST.**

It has long been established that *Vibrio* spp. are autochthonous to the marine, estuarine, and riverine environment. Vibrios cultured from environmental samples commonly lack genes coding for functions associated with pathogenicity for humans and marine animals, e.g., the thermostable direct hemolysin (*tdh*) in *Vibrio parahaemolyticus*. Nevertheless, pathogenic subpopulations of vibrios are potential agents of disease outbreaks and pandemics (7, 19, 23, 37, 44, 50, 65), notably in developing countries, where access to safe drinking water is limited (26, 56), and/or in countries where consumption of raw or undercooked shellfish is common (11, 80). *Vibrio parahaemolyticus* is most frequently associated with gastroenteritis and has been linked to annual outbreaks (7, 8, 44). *Vibrio vulnificus* is more frequently associated with wound infections, with a case fatality rate as high as 50% (5, 10, 27). The abundance and distribution of these two human pathogens have been linked to environmental factors, most notably temperature and salinity, depending on the pathogen and its habitat, and the geographic location (4, 13, 14, 18, 24, 29, 31, 35, 39, 70, 72, 83). Dissolved oxygen (30, 54, 57), chlorophyll (6, 20, 31, 33), and plankton (2, 31, 41, 59, 74) have also been found to be important in the ecology of vibrios. Regulatory authorities responsible for oversight of recreational waters and shellfish harvesting areas employ rainfall, fecal coliform counts, river stages, and, more recently, enterococcus counts to determine opening and closing of specific areas to protect public health (21, 25, 62, 76). Standard microbiological approaches to classification and opening/closing of oyster harvest areas, which are unfortunately not useful for control of exposure to pathogenic *Vibrio* spp., continue to be used and are generally accepted for regulating exposure to other pathogens in the United States (36).

Naturally occurring pathogens, notably vibrios, are ubiquitous in the aquatic environment and contribute to cycling of carbon and other nutrients (24, 61). Clearly, human exposure to these pathogens cannot be completely eliminated, but the incidence of illness can be reduced if environmental conditions that significantly elevate risk can be identified and monitored. Communication of such conditions to stakeholders (regulatory agencies, the shellfish industry, public health officials, at-risk consumers, etc.) would reduce exposure and subsequent disease. An informative, robust system of identification of conditions associated with high risk requires quantifying the association of environmental factors with abundance of total vibrio populations and potentially pathogenic vibrios. Given proven associations as predictors of vibrio abundance, the relevant environmental data can be collected by remote satellite sensing (13, 39, 83).

Development of models to predict presence of vibrio popula-

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tions is facilitated by collecting observations over a range of environmental parameters and recognition that predictive relationships may vary across regions due to differences in ecology (for example, models developed for the Gulf of Mexico may not be applicable to the Pacific Northwest). Furthermore, potentially pathogenic subpopulations of environmental vibrios are not necessarily a constant proportion of the total vibrio population (17, 18, 31, 32, 55, 83). Here we describe an analysis of environmental factors with the potential to improve upon existing predictive models for *V. parahaemolyticus* and *V. vulnificus*. Specifically, we determined densities of total *V. parahaemolyticus* (*tlh*), of potentially pathogenic *V. parahaemolyticus*, as indicated by the presence of the thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin genes (*trh*) (48, 64), and of total *V. vulnificus*, as indicated by the presence of the *V. vulnificus* hemolysin gene (*vvhA*), which has been used as a marker for the species (45, 81, 82). These were determined for water, oyster, and sediment samples collected at sampling stations located in the Pacific Northwest, northern Gulf of Mexico, and Chesapeake Bay.

TABLE 1 Pooled data for WA, GC, and MD according to gene target and sample type

Gene	Sample	Location pools including: PCR/MPN method	Location pools including: DP/CH method
tlh	Water	WA, GC, MD	GC, MD
	Oyster	WA, GC, MD	GC, MD
	Sediment		WA, GC, MD
tdh	Water	WA, GC, MD	
	Oyster	WA, GC, MD	
	Sediment		WA, GC, MD
trh	Water	WA, GC, MD	
	Oyster	WA, GC, MD	
	Sediment		WA, GC, MD
vvhA[a]	Water		GC, MD
	Oyster		GC, MD
	Sediment		GC, MD

[a] *vvhA* data for all samples from WA were omitted.

MATERIALS AND METHODS

Sample collection and processing. From December 2008 to August 2010, water, oyster, and sediment samples were collected in the Pacific Northwest in Hood Canal in Washington (WA), in the northern Gulf of Mexico spanning Louisiana (LA) and Mississippi (MS), and in the Chester River and Tangier Sound of the Chesapeake Bay in Maryland (MD). All samples were collected concurrently in LA, MS, and MD, but in WA, because of logistical problems, oyster and sediment samples were collected intertidally and relayed to a dock, where the water samples were collected subtidally. At all sampling stations, 6 to 12 liters of water, 20 to 25 oysters, and 100 g of sediment were collected and transported to the laboratory in coolers containing ice or ice packs. Water, oyster, and sediment samples were processed as described elsewhere (31). Specifically, water samples were shaken as previously described (1); oysters were scrubbed, shucked, and homogenized; and pore water was decanted from sediment and then diluted 1:1 and shaken as previously described (31).

V. parahaemolyticus and *V. vulnificus* were enumerated as follows. First, 1 ml water, 0.1 g, and 0.01 g oyster and various amounts of sediment (0.0005 to 0.1 g [wet weight]) were spread plated on T_1N_3 agar (1% tryptone, 3% NaCl [pH 7.2]) and VVA agar (2% peptone, 3% NaCl, 1% cellobiose, 0.06% bromthymol blue [pH 8.2]). Then, tdh^+ and trh^+ *V. parahaemolyticus* populations were detected using 1-liter, 100-ml, and 10-ml water enrichments and 10-g and 1-g oyster enrichments in 10× alkaline peptone water (10× APW; 10% peptone, 1% NaCl [pH 8.5]). All samples were incubated at 33°C for 16 to 18 h, as described previously (31).

Enumeration of vibrios. To enumerate *V. parahaemolyticus* and *V. vulnificus* by direct plating and colony hybridization (DP/CH), Whatman 541 ashless filters (Whatman, Kent, ME) were used to lift bacterial colonies from plates, as described elsewhere (31). The filters were probed using alkaline phosphatase-conjugated oligonucleotide probes (DNA Technology A/S, Risskov, Denmark) specific for *vvhA*, *tdh*, *trh*, and *tlh* (31). The DP/CH method is most effective for directly enumerating *tlh* and *vvh* populations, but *tdh* and *trh* populations are often too sparse to rely on DP/CH for enumeration. Therefore, samples were enriched in APW as described above to increase the *tdh* and *trh* populations to higher levels that could be detected and enumerated using the most-probable-number (MPN) method (49). For both total (tlh^+) and potentially pathogenic (tdh^+ and trh^+) *V. parahaemolyticus* populations, serial MPN dilutions were assayed in triplicate for water and oyster enrichments using real-time PCR, as previously described (31, 49). For samples collected in LA and in MS, probes and equipment were used that have been described elsewhere (31). For MD samples, probes were used as described previously (49), and reactions were carried out using an AB 7500 thermal cycler (Applied Biosystems, Carlsbad, CA). For WA samples, a Stratagene Mx300Sp real-time PCR system (Agilent Technologies, Santa Clara, CA) was used for real-time PCR analysis. The *tlh* and internal amplification control (IAC) probes were purchased from Integrated DNA Technologies (Coralville, IA), and the *tdh* and *trh* probes were obtained from Applied Biosystems (Foster City, CA). Each 25-μl reaction mixture consisted of 12.5 μl of 2× Brilliant multiplex quantitative PCR master mix (Agilent Technologies, Santa Clara, CA) and the following reaction components (final concentrations): all three probes at 150 nM, all six primers at 75 nM, and bovine serum albumin (BSA; New England BioLabs, Beverly, MA) at 400 ng/μl. The remainder of the reaction mixtures consisted of 1 μl of the IAC template at the concentration described above, nuclease-free water, and 5 μl of template. The two-step thermal profile employed throughout the study consisted of an initial 9.5-min denaturation step at 95°C, followed by 40 cycles of 30 s denaturation at 95°C and a 45-s combined annealing and extension step at 58°C. Fluorescence data were collected at the end of each amplification cycle. The primer and probe sequences employed were the same as described previously (49).

All sediment samples were analyzed by DP/CH alone to enumerate total and pathogenic vibrios in sediment. The PCR/MPN method was not used because sediment has previously been found to contain very high levels of all four gene targets, and they could therefore be effectively enumerated using DP/CH; in addition, PCR analysis of sediment samples has proven unfruitful in our experiences (data not shown). *V. vulnificus* levels were consistently low in the Pacific Northwest; among the 174 water, oyster, and sediment samples collected there during this study, *V. vulnificus* was detected in only one water and one sediment sample. Thus, determinations of *vvhA* densities in WA samples were excluded from statistical analyses. In addition, during analyses of samples from the Pacific Northwest using the DP/CH method, cross-reactivity with either another *Vibrio* species or an unknown bacterium was noted. *V. parahaemolyticus* *tdh* and *trh* data from oyster and water analyses were therefore excluded, and only PCR/MPN data from WA were used for water and oyster samples. To correct for possible cross-reactivity at other sites, the DP/CH data were excluded from the analyses for the GC and MD sites when *tdh* and *trh* population densities were determined. The resulting data pools are listed in Table 1.

Environmental parameters. At each collection site, water temperature and salinity were measured at the surface and bottom using a digital handheld conductivity meter (model 30-25FT; Yellow Springs Instruments, Yellow Springs, OH). Chlorophyll *a* was measured by high-performance liquid chromatography at the University of Hawaii as follows.

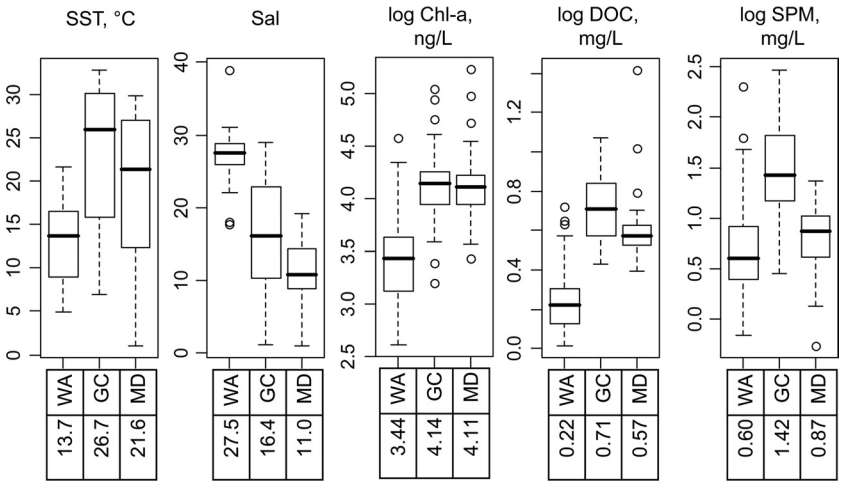

FIG 1 Box plots of sea surface temperature (SST), salinity (Sal), chlorophyll *a* (Chl-*a*), dissolved organic carbon (DOC), and SPM (suspended particulate matter) in samples from Washington (WA), Mississippi and Louisiana (Gulf Coast, GC), and Maryland (MD). Box plots summarize distribution by indication of the maximum, 75th percentile, median, 25th percentile, and minimum values. Additional circles indicate outlier values identified by the statistical package R. Points more than 1.5 times the interquartile range above the third quartile or below the first quartile were plotted individually as outliers. Median values are below the graphs.

Triplicate volumes of up to 200 ml were filtered using 25- to 47-mm diameter GF/F filters (Whatman, Kent, ME). Filters were stored at −20°C until shipped overnight on dry ice to the University of Hawaii, where concentrations of chlorophyll *a* (Chl-*a*) were measured in methanol extracts on a Cary model 50 UV–visible-light spectrophotometer, as described previously (38). Suspended particulate matter (SPM) was measured by weighing predried GF/F filters using a high-precision scale and filtering up to 200 ml water; the filters were dried overnight at 65°C and reweighed.

To determine dissolved organic carbon (DOC) concentration, triplicate water samples were prefiltered using a 0.45-μm Gelman hydrophilic polypropylene Acrodisc syringe filter (Pall, Ann Arbor, MI) and treated with HCl to convert inorganic carbon to CO_2. The samples were stored at −20°C until they were analyzed as follows. CO_2 from inorganic carbon was manually purged from samples by adding additional HCl followed by overnight incubation at room temperature. Total organic carbon was measured using a Shimadzu TOC-V CSN carbon analyzer equipped with an ASI-V autosampler (Shimadzu Scientific Instruments, Columbia, MD).

Statistical analyses. Multilevel generalized linear mixed models (GLMM) were used to estimate the distribution of vibrio abundance in oyster, sediment, and water and the relationship between abundance and environmental predictors. Underlying (latent) distributions of vibrio abundance were assumed to be lognormal, with mean $\log_{10}$ densities generally being presumed to be linearly related to environmental parameters that were considered predictors of abundance. However, given the wide range of salinities observed across sampling locations and consequent likelihood of a nonlinear dependence, a quadratic polynomial was used to model the effect of salinity. Estimates of location and scale of latent distributions of abundance for each combination of gene target, sample type, and sampling location were obtained by fitting null (intercept-only) models with no predictor variables. To facilitate identification of associations between abundance and environmental predictors weakly identified when each sampling location was considered separately, data were pooled across sampling locations for each combination of gene target and sample type. Raw plate count and real-time PCR/MPN observations for multiple aliquots and dilutions of the same sample were treated as repeated and discrete-valued measurements of the same underlying abundance in the given sample. Raw observations comprise the response variables of GLMM regression, with plate counts and PCR/MPN outcomes at each dilution level being treated as independent Poisson and binomial outcomes, respectively, conditional on latent distribution of abundance and volume of sample examined in each aliquot or dilution. Given the apparent inhibition of the PCR at low dilutions in some samples, the PCR/MPN data were truncated to one dilution, as described elsewhere (31). In regression analyses, temperature and salinity parameters were expressed in degrees Celsius and parts per thousand, respectively, while Chl-*a*, DOC, and SPM were expressed in base 10 logarithms of their respective measurement units. GLMM regression parameter estimates were determined by Markov chain Monte Carlo (MCMC) sampling of posterior Bayesian distributions, conditional on the observed data and noninformative prior distributions. Associations between vibrio abundance and environmental parameters were summarized using McKelvey and Zavoina's pseudo-R^2 (28, 67), as a measure of the proportion of variation in latent distributions of abundance attributable to variation in the environmental parameter. Statistical analyses were conducted using WinBUGS (40) and the R2WinBUGS package of R (58, 71). Statistical significance of associations was assessed by identifying Bayesian 95% credible intervals for regression parameters that were exclusive of zero (51).

For graphical presentation of data, the numbers of *vvhA*$^+$, *tlh*$^+$, *tdh*$^+$, and *trh*$^+$ vibrios were determined by dividing the total number of CFU on one or more plates by the corresponding total volume of water or weight of oyster and sediment examined. Only counts between 1 and 250 CFU per plate were plotted. Therefore, the limit-of-detection (LOD) ranges for *V. vulnificus* in water, oysters, and sediment were 1 to 250 CFU/ml, 10 to 25,000 CFU/g, and 100 to 83,333 CFU/g, respectively, because 1 ml water, 0.1 to 0.01 g oyster, and 0.01 to 0.003 g sediment were tested per *V. vulnificus* plate and only 1 to 250 CFU were counted per plate. The LODs for *V. parahaemolyticus* in water, oysters, and sediment were 1 to 250 CFU/ml, 10 to 25,000 CFU/g, and 20 to 83,333 CFU/g, respectively, because 1 ml water, 0.1 to 0.01 g oyster, and 0.05 to 0.003 g sediment were tested per *V. parahaemolyticus* plate.

RESULTS

Environmental parameters. Sea surface temperature ranges across the four sample sites were relatively similar (Fig. 1). The lowest temperatures were measured in WA, with LA and MS temperatures being highest and MD samples showing the widest temperature range. Based on their similar geography and climate, the two Gulf Coast sites, LA and MS, were combined (GC) for analyt-

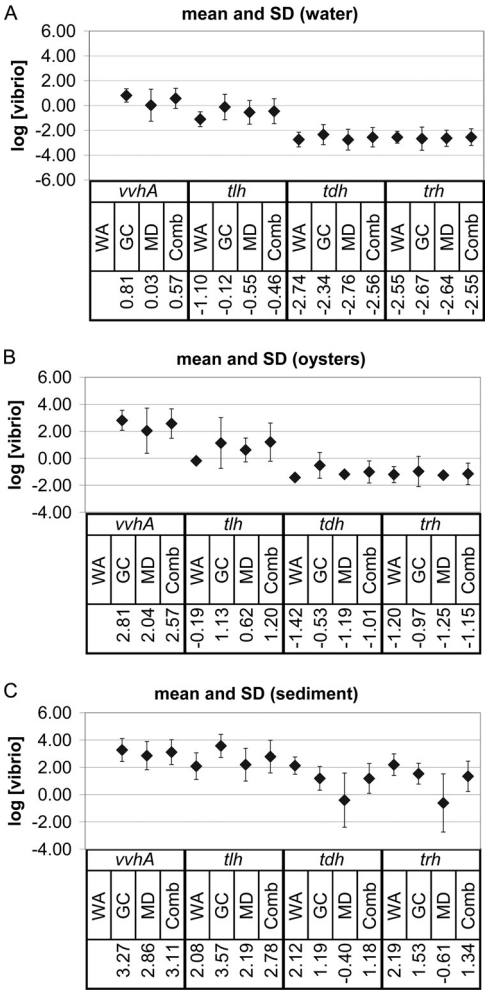

FIG 2 Model-based estimates of mean and standard deviation (SD) of log abundance by location and gene target. Estimates of mean and SD of log CFU/ml (water) and log CFU/g (oysters and sediment) are based on measurements from DP/CH (all *vvhA* populations and sediment data), from real-time PCR/MPN (*tdh* and *trh* populations in all water and oyster samples, and *tlh* in WA water and oyster samples), or from both (*tlh* populations in GC water and oyster samples and in MD water and oyster samples). Values are means with standard deviations of the distributions (not standard errors of the means).

ical and reporting purposes. Salinities were highest for WA samples and lowest for MD samples; GC samples had the widest salinity range. WA samples contained the lowest median Chl-*a* concentration, median DOC, and SPM, and GC samples had the highest medians for the three parameters.

Model-based estimates of abundance. A large number of negative results were obtained using the DP/CH method for quantitation of *tdh* and *trh* populations in water samples and in oysters. It was concluded, therefore, that the DP/CH approach is not as informative as the MPN approach for enumeration of *tdh* and *trh* populations, and these data were excluded for this reason. Results for the WA samples showed *vvhA* populations to be very low in all three sample types, an indication that *V. vulnificus*, if present, was below the limit of detection levels in agreement with previous studies (34). At all sampling sites, the largest numbers of vibrios (*tlh*, *tdh*, *trh*, and *vvhA* organisms) were obtained when the water temperature was high (Fig. 2). All GC samples had high *tlh* and *vvhA* population densities and temperatures. The GC samples also had the highest *tdh* and *trh* population densities in oysters, but the WA samples had the highest *tdh* and *trh* population densities in sediment. Overall, trends in vibrio population numbers in water were similar for the all sample types, but WA samples exhibited low *V. vulnificus* densities. MD samples exhibited the lowest mean vibrio densities, with respect to the *tdh* and *trh* gene targets, with relatively large standard deviations.

Intrasample comparisons of *V. parahaemolyticus* and *V. vulnificus*. Densities of *V. parahaemolyticus* (*tlh*) were compared to those of *V. vulnificus* (*vvhA*) on a sample-by-sample basis; i.e., each *tlh* abundance was compared to *vvh* abundance in the same sample. Comparisons summarizing relative abundance in CFU/ml of water or CFU/g of oyster or sediment were used to infer prevalence of one species over the other across sample type and temperature range (Fig. 3). Data are presented in this fashion due to the relatively high rate of nondetection, making calculation of percentages problematic on a sample-by-sample basis. For LA samples, when *tlh* and *vvhA* populations were detectable by DP/CH, *tlh* organisms outnumbered *vvhA* organisms in most samples (Fig. 3). Specifically, *tlh* organisms outnumbered *vvhA* organisms about two-thirds of the time in water and sediment and about four-fifths of the time in oysters. The reverse was observed for MS and MD samples, where *vvhA* organisms typically outnumbered *tlh* organisms. Thus, overall, in LA samples, *V. parahaemolyticus* was dominant more frequently in all sample types than *V. vulnificus*, whereas MS and MD samples were more often dominated by *V. vulnificus* than by *V. parahaemolyticus*.

Environmental predictors of abundance. Multilevel (GLMM) regression models and associated measures of relative importance of predictor variables (pseudo-R^2 values) were applied to data pooled across sampling locations to assess the proportion of variation in vibrio abundance attributable to variation in each environmental parameter. Where identified as statistically significant, DOC accounted for 13% of *tlh* population variability in oysters (Table 2), 15% of *tdh* population variability in water, and 12% of *trh* population variability in sediment; its impact on the other factors was insignificant. Where identified as statistically significant, Chl-*a* accounted for 5% of *tlh* population variability in sediment, 22% of *tdh* population variability in sediment,13% of *trh* population variability in oysters, and 9.8% of *trh* population variability in sediment. Similarly, where identified as statistically significant, SPM accounted for 6 to 29% of variability in vibrio abundance, depending on sample type, with the abundance of *tdh* organisms in oysters being the highest. Salinity accounted for 9% of *tlh* population variability in oysters and 3.7% of *tlh* population variability in sediment. Although the pseudo-R^2 value for salinity in Table 2 was high for *tdh* in the water column (31%), this value was not statistically significant and most probably due to chance. SST accounted for 7.1 to 34% of *V. vulnificus* and *V. parahaemolyticus* variability and was a strong predictor in all samples except for *tdh* populations in water and oysters and *trh* populations in oysters. In most sample types for which SST was a significant predictor, SST explained a larger percentage of variability than any of the other parameters measured. In instances where SST was not significant, SPM was the strongest predictor (Table 2). DP/CH detection rates were highest in sediment samples, followed by oysters and water, and the highest *tdh* or *trh* nondetection rates were in water and in oysters (Table 3).

Estimates determined by analysis of data pooled across sam-

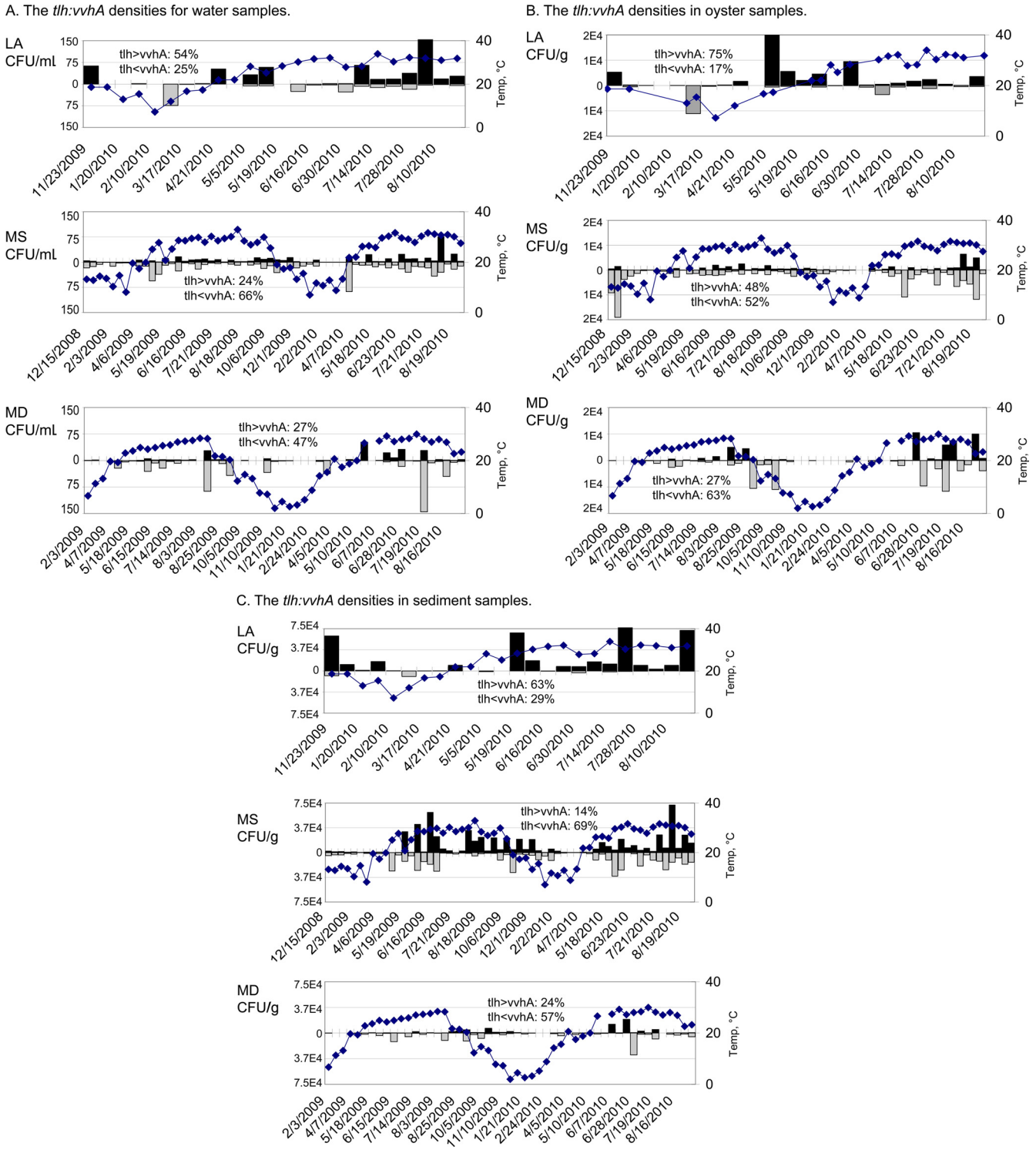

FIG 3 Relative densities of *tlh* and *vvhA* populations by habitat and location. The DP/CH-derived *tlh* population densities were compared to DP/CH-derived *vvhA* population densities on a sample-to-sample basis for water (A), oysters (B), and sediment (C). Black bars, *tlh* population densities; gray bars, *vvhA* population densities; lines with diamonds, sea surface temperature (°C) plotted on the secondary (right) *y* axis. WA data were excluded from these graphs because of the lack of *vvhA* organism counts.

pling locations were further evaluated by comparing to results of analyses of unpooled data, considering each site separately. Analysis of unpooled data was conducted to assess consistency of identified associations in the pooled analyses across each sampling location. Similar patterns of association were observed in analysis of unpooled data as in the analysis of pooled data, but the uncertainty of the relationships identified was much greater. For the parameters SST and SPM, which exhibited a relatively strong as-

TABLE 2 Relative importance of environmental parameters for all sampling locations combined

	Relative importance[a]											
	tlh			*vvhA*			*tdh*			*trh*		
Parameter	Water	Oyster	Sediment	Water	Oyster	Sediment	Water	Oyster	Sediment	Water	Oyster	Sediment
Log DOC	1.88	**13.1**	0.96	1.56	2.05	4.36	**15.3**	4.17	5.05	7.77	5.89	**12.4**
Log Chl-*a*	1.98	0.63	**5.01**	1.29	2.55	1.38	12.1	6.31	**22.0**	3.11	**12.9**	**9.80**
Log SPM	**15.6**	**5.99**	**7.40**	2.38	2.80	3.80	**22.9**	**28.9**	1.91	8.64	**17.4**	**10.1**
Sal	2.45	**8.98**	**3.70**	3.56	3.72	2.60	30.7	5.24	4.17	5.78	4.72	4.79
SST	**11.0**	**18.0**	**34.3**	**11.5**	**27.6**	**9.00**	4.65	3.65	**11.2**	**14.2**	7.72	**7.05**

[a] Relative importance is based on pseudo-R^2 statistics derived from GLMM analysis. Sal, both linear and quadratic terms for salinity combined. Values in bold have a *P* value of <0.05.

sociation with vibrio abundance, the estimated effect size (magnitude and sign of regression coefficients) across sampling locations was more consistent with that of the pooled analysis than was the case with other parameters.

DISCUSSION

A major goal of our group is to develop ecological models that can be used in conjunction with remotely sensed data collected from and applicable to different geographic regions of the world (i.e., algorithms such as that found at http://www.eol.ucar.edu/projects/ohhi/vibrio/). Development of ecological models for bacteria is strengthened by collection and analysis of samples from diverse geographic locations. Inclusion of geographically distinct study sites to maximize understanding of the role of environmental parameters is a unique contribution of this study. An additional strength is the length and intensity of sampling, which included 594 water, oyster, and sediment samples collected weekly to biweekly over 21 months and a range of environmental parameters measured; this was one of the longest and most intensive sampling programs associated with vibrio abundance and distribution (15, 18, 22, 43, 46, 53, 54, 73, 78, 83). Furthermore, our sampling was carried out year-round to examine seasonal variations in vibrio densities. Environmental factors associated with incidence and geographic distribution of *V. parahaemolyticus*, potentially pathogenic *V. parahaemolyticus*, and *V. vulnificus* at four sampling locations in three U.S. coastal areas were analyzed.

TABLE 3 Summary statistics for *V. parahaemolyticus* and *V. vulnificus* densities

Sample	Probe target[a]	Density range (CFU/ml or CFU/g) (median)[b]	DP/CH detection rate (%)
Water	*vvhA*	<1 to >250 (6.0)	79.2
	tlh	<1 to 204 (1.5)	69.5
	tdh	<1 to 66 (<1)	18.1
	trh	<1 to 39 (<1)	19.7
Oysters	*vvhA*	<10 to >2.5E4 (673.9)	86.3
	tlh	<10 to 2.2E4 (186)	81.5
	tdh	<10 to 241 (<10)	24.8
	trh	<10 to 982 (<10)	34.9
Sediment	*vvhA*	<100 to >8.3E4 (525)	61
	tlh	<20 to >8.3E4 (715)	89.7
	tdh	<20 to 2.4E3 (25)	61.3
	trh	<20 to 3.5E3 (50)	64.2

[a] *tlh,* thermolabile hemolysin; *tdh,* thermostable direct hemolysin; *trh, tdh*-related hemolysin; *vvh*, *V. vulnificus* hemolysin.
[b] DP/CH was used to determine densities in water (CFU/ml), oysters (CFU/g), and sediment (CFU/g).

Ranges of vibrio densities were wider and detection rates were higher in this study than in our previous study, as were ranges of environmental parameters (31). The current study identified the highest SPM levels on the Gulf Coast, a result that was not surprising, since the Mississippi River plume contributes to turbidity and eutrophy (http://earthobservatory.nasa.gov/IOTD/view.php?id=4982; last accessed 4 July 2012), and southerly wind events frequently resuspend sediment in the shallow waters of the northern Gulf of Mexico (75). There appeared to be a degree of niche-specific sequestering, as evidenced by the fact that *vvhA* populations exhibited the highest detection rates in oysters while *tlh* populations exhibited the highest detection rates in sediment. Thus, *V. parahaemolyticus* and *V. vulnificus* differ in their niches. In addition, the intrasample dominance of *tlh* organisms in samples from LA compared to that of *vvhA* organisms in MS and MD samples indicated some state-to-state variability that may merit consideration as model-based risk assessments are further developed; i.e., *vvhA* organisms did not consistently outnumber *tlh* organisms on a within-sample basis, and in the current study this ratio varied by geographic location.

By extending previous work on both vibrio ecology and ecological models for prediction of *Vibrio* sp. abundance in the aquatic environment, it was reaffirmed that temperature is a strong predictor of abundance and distribution of total vibrios (3, 4, 30, 31, 33, 42, 52, 54, 63, 74, 77), and this is particularly useful in the warmer Gulf Coast states included in this study (LA and MS). Though it is clear that temperature is dominant, there is no specific hierarchy among the parameters; environmental factors interact to influence vibrio abundance, but precise details of all such variables and how they interact have yet to be fully described.

Despite its dominance with respect to *tlh* and *vvhA*, SST was not a strong predictor for densities of vibrios with the pathogenicity genes *tdh* and *trh* in this study. This finding suggests that environmental factors may differentially affect the abundance of pathogenic subpopulations. This is particularly relevant given previous observations that the percentage of total *V. parahaemolyticus* containing these *tdh* and/or *trh* genes appears to be variable and inversely related to temperature (18, 19, 83).

Observed associations between abundance and salinity were minimal despite the relatively wide salinity range of this study. Salinity correlated significantly only with the presence of *tlh* in oysters and sediment, not with any other measurements in the analysis of data pooled over sampling locations. This was unex-

pected, given previous observations of significant correlations between salinity and vibrios in samples from Mississippi and Alabama (31). This finding did not appear to be an artifact of the pooled data analysis, as analyses of unpooled data by sampling location separately were generally consistent. Specifically, although effects of salinity did not follow the same nonlinear (quadratic) relationship at each sampling location (e.g., due to narrow range of salinity above or below an optimum), the apparent effects at each sampling location were consistent with that of the pooled data, even when the effect overall was identified as not statistically significant. Regression models incorporating an interaction between temperature and salinity were explored but did not significantly improve overall goodness-of-fit or otherwise provide an interpretation for the unexpected findings.

Some studies have identified a significant relationship between vibrios and salinity (6, 12, 30, 31, 60, 69, 79), while others did not (47, 60, 66, 68), so the relationship with salinity may be variable and complex. For example, Griffitt and Grimes (unpublished data) found that large salinity shifts, as seen during the opening of the Louisiana Bonnet Carré Spillway following the Mississippi River floods of 2011, can cause detectable and significant change in the relative numbers of pathogenic vibrios. *In vitro* growth rates of *V. vulnificus* biotypes 1, 2, and 3 (9) have been related to distance from shore (47) with respect to salinity and analyzed to determine the relationship of salinity of the coastal ocean and estuaries to vibrio abundance and distribution. Differences between studies may also be attributable to different salinity ranges or other factors such as the nutrients sparing the salinity requirement for growth (66).

The statistically significant contributions of chlorophyll and DOC to the vibrios in this study were minimal, but findings for SPM were suggestive of a stronger effect. This is consistent with the significant relationships previously identified between turbidity and *V. parahaemolyticus* abundance (30, 31, 33, 47, 54). A positive association with turbidity is consistent with expectations because vibrios, like many other bacteria, are frequently attached when in the aquatic environment (16). A higher density of particulate matter suspended in the water column logically provides habitat for a greater density of vibrios. The present study represents initial efforts to quantify that relationship.

It was surprising that SST was the only factor that was a statistically significant predictor of *vvhA* population density in any sample type, even when the paucity of *vvhA* organisms in Washington was accounted for. We and others previously demonstrated relationships between the abundance of *vvhA* organisms and environmental parameters, including temperature, salinity, and chlorophyll (31). Also interestingly, WA samples exhibited the lowest median Chl-*a*, DOC, and SPM levels, as well as the highest salinities.

The proportions of *tdh* and *trh* populations in the Pacific Northwest as measured by DP/CH were high compared to that of total *V. parahaemolyticus* (i.e., tlh^+ organisms), suggesting that tdh^+ and/or trh^+ *V. parahaemolyticus* is present in very large numbers. It was concluded that the relatively high salinities in the Pacific Northwest were unrelated to the high rates of detection of *tdh* and *trh* populations, because similar salinities observed at the other sampling sites in this study were not associated with high rates of detection of these populations (data not shown). To investigate the possibility of cross-reactivity with other vibrios in the Pacific Northwest, a small subset of vibrios in the GC collection was queried. Of the 23 vibrios containing *trh*, only two were identified as *Vibrio alginolyticus*, with the remaining 21 identified as *V. parahaemolyticus*; *tdh* was found only in *V. parahaemolyticus* (Rachel Clostio, personal communication).

Other studies of WA tdh^+ and trh^+ strains, including strain genotyping (R. N. Paranjpye, W. B. Nilsson, R. G. Lillie, O. S. Hamel, and M. S. Strom unpublished data) and both multilocus sequence typing and complete genomic sequencing (Turner et al., unpublished data) demonstrate that strains in the Pacific Northwest carrying *tlh*, *tdh*, and *trh* are indeed *V. parahaemolyticus*. Thus, the explanation for the high rates of detection of *tdh* and *trh* populations in the Pacific Northwest as measured by DP/CH remains unknown.

The unforeseen need for the asymmetrical treatment of DP/CH results from Washington due to unexpectedly high rates of *tdh* and *trh* DP/CH results was deemed acceptable and thought to contribute minimal artifacts, because both DP/CH and PCR/MPN methods target the same genes (49). The PCR/MPN method does include additional regions of specificity by its nature, because it includes two oligonucleotide primers and a fluorescent probe, while DP/CH includes only an alkaline phosphatase-conjugated probe that binds to the region targeted by the forward PCR primer. However, potential variability and artifacts were minimized by treating all three sites in the same manner where possible, i.e., including only PCR/MPN results for *tdh* and *trh* gene targets.

Future studies will address the impact of individual parameters on vibrio abundance, for which microcosm studies have been initiated at the University of Maryland that address molecular genetic determination of the vibrios indigenous to the respective geographic regions of this study, and these results will be presented elsewhere. Additional data will also be analyzed as a result of a recently concluded concurrent sampling regimen in the four sampling states. A focus of analysis of these data will be exploring possible differences in relationships between vibrio abundance and predictor variables across sampling locations, and this will provide further insight about the initial assessment based on pooling of data. Findings from the microcosm study will be evaluated to better inform model selection in the analysis of field study observations. Zooplankton and phytoplankton densities and relationships with additional pigments indicative of phytoplankton will also be analyzed. A sufficiently large complement of data will facilitate identification of statistical models that both are interpretable and provide the best possible predictive value.

In conclusion, the microbial ecology of selected *Vibrio* spp. has been extensively studied to determine the importance of specific environmental parameters influencing the incidence, distribution, and abundance of total and pathogenic vibrios. This study builds upon existing data sets and findings by including an exceptionally wide range of geographic regions, vibrio densities, seasons, and environmental parameters not studied previously. Maximizing the size of the study made it possible to study parameter ranges that cannot be investigated by studying only a single study site. This study confirmed some previously reported findings (e.g., the impact of temperature) but also identified some new findings (e.g., the differences in the strength of correlation of *V. parahaemolyticus* and *V. vulnificus* densities to environmental parameters). Diversifying the geographic niches included in this study improves the chances of identifying environmental signatures that

can be used to predict and possibly prevent vibrio outbreaks in a wide and possibly global range of geographic locations.

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